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Heat-hardening effects on mating success at high temperature in *Drosophila melanogaster*

L. Stazione[a,b], F.M. Norry[a,b], P. Sambucetti[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Ecología, Genética y Evolución, Buenos Aires, Argentina
[b] CONICET, Universidad de Buenos Aires, Instituto de Ecología, Genética y Evolución (IEGEBA), Buenos Aires, Argentina

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ABSTRACT

Reproduction is strongly influenced by environmental temperature in insects. At high temperature, mating success could be influenced not only by basal (non-inducible) thermotolerance but also by inducible plastic responses. Here, mating success at high temperature was tested in flies carrying contrasting genotypes of heat resistance in *Drosophila melanogaster*. The possible heat-hardening effect was tested. Mating success did not differ between heat-resistant and heat-sensitive genotypes when tested both at high (33 °C) and benign (25 °C) temperature, independently of the heat-hardening status. Importantly, heat-hardening pre-treatment increased in a 70% the number of matings at 33 °C in a mass-mating experiment. Further, mating latency at 33 °C was shorter with heat hardening than without it in single-pair assays Heat-hardening had previously been showed to improve short-term thermotolerance in many organisms including *Drosophila*, and the present results show that heat hardening also improve mating success at elevated temperature. Previous exposures to a mild heat stress improve short-term mating success as a plastic response of ecological relevance. Such heat-hardening effects on mating success should be relevant for predicting potential evolutionary responses to any possible current scenery of global warming, as well as in sterile insect release programs for pest control in elevated temperature environments.

1. Introduction

Environmental temperature is a well known stress agent affecting the distribution and reproduction of species in nature (Hoffmann and Sgro, 2011; Franks and Hoffmann, 2012), with a growing interest under a predicted scenario of increasing global temperature (Deutsch et al., 2008; Kellermann et al., 2009; Kelly et al., 2012; Van Heerwaarden et al., 2016). As reproductive success is the most inclusive measure of overall fitness (Brooks and Endler, 2001), reproductive fitness main components such as mating success are of special interest in thermal adaptation studies. However, few studies have directly tested for links between mating success and thermotolerance genotypes at elevated temperature (Loeschcke et al., 2011).

Mating success at high temperature was recently shown to be affected by thermal-stress selection in *Drosophila* (e.g., Dolgin et al., 2006; Sambucetti and Norry, 2015). Loeschcke et al. (2011) have found that alternative genotypes for a major quantitative trait loci (QTL) affecting heat resistance in the middle of chromosome 2 predict mating success and the ability to locate a food and reproductive resources in *D. melanogaster* at high temperature. Another large-effect QTL for heat resistance in this model insect was identified in the middle of chromosome X (Norry et al., 2004, 2007, 2008; Rand et al., 2010; Arias et al., 2012), but whether this X-linked QTL also affects mating success at high temperature is still unknown.

Adaptation to changing climatic conditions depends not only on basal but also on inducible thermotolerance traits (Hoffmann and Parsons, 1991; Hoffmann et al., 2003; Hoffmann and Daborn, 2007; Reusch and Wood, 2007; Sgrò et al., 2016). For instance, inducible thermotolerance is the increase in heat resistance due to pre-exposition to a sub-lethal thermal stress, as in the case of heat-hardening and heat acclimation (e.g., Hoffmann et al., 2003). In insects, the adaptive response to heat hardening (i.e., short-term inducible thermotolerance) is a plastic response representing ecologically relevant phenotypes for adaptation to thermal environments (Hoffmann et al., 2002, 2003; Sørensen et al., 2003; Rako and Hoffmann, 2006; Sgrò et al., 2016). *D. melanogaster* is found over wide geographical areas from tropical to temperate regions and adaptive phenotypes of inducible thermotolerance can be genetically variable both within populations and

* Corresponding author at: Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Ecología, Genética y Evolución, Buenos Aires, Argentina.
E-mail address: pablosambucetti@ege.fcen.uba.ar (P. Sambucetti).

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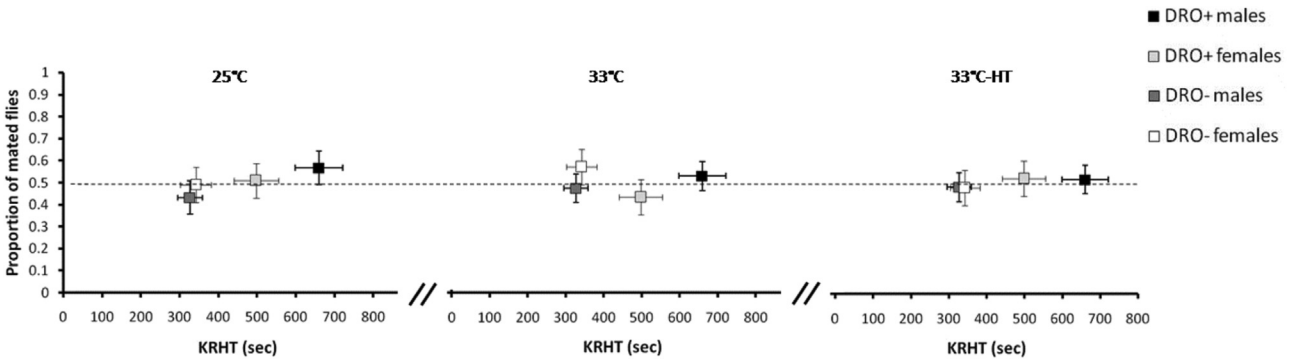

Fig. 1. Proportion of mating flies relative to the total number of copulates observed, averaged over replicates of mass-mating cages (± standard error) at 25 °C, 33 °C and 33 °C after a heat hardening pre-treatment (HT), is shown for both heat-resistant (DRO +) and heat-sensitive (DRO −) stocks. Values are shown as function of the knockdown resistance to high temperature at 37 °C (mean KRHT ± standard error) of each stock.

between populations. As a result of heat hardening (reviewed in Hoffmann et al. (2003); Bowler and Terblanche (2008)), heat resistance usually increases by previous exposure to high temperature (Krebs and Loeschcke, 1994; Loeschcke and Hoffmann, 2007). However, the effect of this plastic response on mating success has been rarely studied (see Jørgensen et al. (2006)).

Because *D. melanogaster* is a cosmopolitan insect, selection can act favoring alternative genotypes with effects on thermal performance in different environments. It is expected that genotypes from warm environments should thus have thermal optima at higher temperatures than genotypes from cold environments (Bowler and Terblanche, 2008; Angilletta et al., 2009). Recombinant inbred lines (RIL) can be used to generate lines with contrasting genotypes of a thermotolerance-QTL, particularly if RIL were generated from crosses between parental stocks that had strongly diverged for thermotolerance (Loeschcke et al., 2011; Stazione et al., 2017). Previous studies in *D. melanogaster* identified several thermotolerance-QTLs (Norry et al., 2004, 2007, 2008; Morgan and Mackay, 2006; Arias et al., 2012; Takahashi et al., 2011; Sambucetti et al., 2013). Laboratory lines that differ in genotypes of a thermotolerance-QTL can be set up by crossing subsets of RIL according their genotypes. Such fly stocks can be used to test the possible effect of thermotolerance-QTLs on mating success at elevated temperature both in heat-hardened and non-hardened flies, as done for other traits (Loeschcke et al., 2011; Stazione et al., 2017). Here, we tested the effects of an X-linked thermotolerance QTL (previously identified in Norry et al. (2007, 2008)) on mating success at high temperature in *D. melanogaster*. We used recombinant flies carrying contrasting genotypes for the above mentioned thermotolerance QTL, obtained from a subset of RIL that exhibited high variation not only for heat knockdown resistance (Norry et al., 2008) but also for heat-stress survival in field experiments (Borda et al., 2018). We tested the effects of a heat-hardening treatment on mating success in these lines, as there is considerable variation for heat-hardening effects on heat knockdown resistance (Norry et al., 2008). First, we examined whether the heat-resistant QTL genotype exhibits higher mating success than the heat-sensitive QTL genotype at high temperature in a mass-mating experiment. Second, we examined any possible beneficial effects of a heat hardening on copulatory success in our mass-mating experiment. Finally, we scored mating latency in single-pair assays (i.e., the time elapsed from the release of one virgin fly of each sex until mating within a vial), both at 25 °C and 33 °C with and without heat hardening, to test for short-term hardening effects in a context free of any possible male-male interactions.

2. Materials and methods

2.1. Fly stocks

Stocks in this study were obtained from crosses between subsets of recombinant inbred lines (RIL) derived from parental lines selected for high and low knockdown resistance to high temperature (KRHT). Selection of parental lines was performed using a knockdown tube at 36.5 ± 0.2 °C (Norry et al., 2004). The high KRHT selected line was derived from an Australian natural population and denoted as SH2 line whilst the low KRHT selected line was derived from a Danish population and denoted as D48 line (Norry et al., 2008). F1 females (from SH2 × D48 cross) were backcrossed to males from each parental line to establish a RIL panel from the D48 backcross (RIL-D48) and a RIL panel from the SH2 backcross (RIL-SH2). RIL were obtained by full-sib mating for 15 consecutive generations and subsequently genotyped for thirty six microsatellite locus spread throughout all three major chromosomes (Norry et al., 2008). Microsatellite locus DROSEV (bands 10A1–10A2) is a genetic marker closely-linked to a KRHT QTL identified on chromosome X (10A-12D) (Norry et al., 2007, 2008). A subset of RIL were chosen and crossed to establish two fly stocks used in this study, a heat-sensitive and a heat-resistant stock as previously described in Stazione et al. (2017), resulting in two stocks that strongly differ in KRHT for about 150 s in females and 300 s in males (Fig. 1). Specifically, five RIL-SH2 (lines 38; 49; 53; 68; 148) and five RIL-D48 (lines 8; 32; 50; 57; 89; 157) were crossed to set up the heat-sensitive stock. This stock was fixed for the DROSEV marker allele from the low KRHT line D48 (QTL allele conferring low heat resistance) and denoted as DRO − . In addition, four RIL-SH2 (lines 16; 32; 81; 82) and four RIL-D48 (lines 31; 35; 72; 98) were crossed to set up the heat-resistant stock. This stock was fixed for the DROSEV marker allele from the high KRHT line SH2 (QTL allele conferring high heat resistance) and denoted as DRO + . Thus, DRO − and DRO + stocks were fixed for the D48 and SH2 DROSEV marker allele, respectively, with the rest of the genome being both polymorphic and recombinant between the D48 and SH2 parental chromosomes. Recombination between DROSEV and adjacent markers is expected to increase with distance to DROSEV (bands 10A1–10A2), whereas DRO − and DRO + were fixed for recombinant parental alleles at adjacent markers (i.e., marker X11347407ca at band 10C3 was fixed for D48 allele in all eight RIL used to construct DRO + whereas this marker was fixed for SH2 allele in all ten lines used to set up DRO − ; marker DMU96440 at band 7B3 was fixed for D48 allele in all eight lines used to construct DRO + whereas this marker was fixed for SH2 allele in all ten lines used to construct DRO − , Stazione et al., 2017). The stocks obtained from these crosses differ significantly for KRHT (Fig. 1), with the DRO + stock being approximately 1-fold and 0.6-fold more heat resistant than the DRO − stock for males and females respectively (Stazione et al., 2017).

DRO− and DRO+ stocks were initially set up each one with 10 males plus 10 females from each one of the above mentioned RIL, in two 125-ml bottles containing 40 ml of instant mashed potatoes plus water and nipagin as culture medium (hereafter referred to as standard bottles). Stocks were maintained at 25 ± 1 °C in a 12:12 h light/dark cycle in five replicated standard bottles for 10 consecutive generations of random mating. Flies from each stock were mixed among replicated cultures every generation to allow free recombination of the genome except for the fixed QTL alleles as described above.

2.2. Mating success assays

Experimental flies were reared in standard bottles by placing 15 males plus 15 females per bottle from each stock, with 2–3 standard bottles per stock. Bottles were placed at 25 °C under 12 h ligth/12 h dark cycle. Flies were allowed to lay eggs for four days and after that removed from the bottles. Virgin flies emerging from these bottles (collected within 8 h) were sexed under slight CO_2 anesthesia and placed in standard vials with fresh food for four days previous to each mating assay.

Mating assays were firstly carried out through competition for mating between DRO+ and DRO− virgin flies at three different thermal treatments in a walk-in incubator room: 25 ± 1 °C, 33 ± 1 °C and another mating experiment at 33 ± 1 °C after a heat hardening pre-treatment (HT). This heat hardening pre-treatment (HT) was carried out by exposing flies at 30 ± 1 °C for four hours during the first three days of the adult life (pilot assays showed that this level of stress is not lethal, which is found as a moderate stress in some natural environments, as in Loeschcke et al. (2011) and Borda et al. (2018)). Mating success was measured in flies of four days of age which were released within transparent plastic cages (20 × 12 × 10 cm) with a thin cloth net as lid to allow gas exchange from the environment. Two small dishes (2 cm, dia) containing standard food media plus dehydrated yeast were placed inside the mating cages to stimulate courtship and mating. Forty flies (20 males plus 20 females) of each stock (DRO+ and DRO−) were released into the cages, making a total of 80 flies per cage. Before releases, flies were transferred to vials with 0.0015 (± 0.0005) g of fluorescent micronized dust and lightly shaken. Dust colours were randomly assigned to the different stocks and changed between cages. Flies were observed during the next 4 h after releases between 13:00 and 17:00 h. Mating pairs were collected from the cage by using an aspirator tube. Each collecting pair was placed into an empty vial and subsequently frozen to preserve colorants for posterior identification of stock origin under 10X magnification. Cages were replicated between 6 and 10 times for each thermal treatment. Finally, we scored mating latency in single-pair assays as the time elapsed from the release until copulation of a virgin fly of each sex within a standard vial (11 × 2 dia cm) containing culture medium plus dehydrated yeast. To set up these single-pair assays, newly-emerged flies were sexed under slight CO_2 anesthesia, transferred to single-sex vials at 25 °C and aged to have 4 days old for each thermal assay. A subset of these flies received the heat-hardening treatment described above. We scored mating latency within the first two hours, when more than 85% of flies mated. This experiment was run both for DRO+ and DRO− flies, separately, for the three thermal treatments described above for the mass mating assay, with 50–60 flies of each sex per thermal treatment and fly stock.

2.3. Statistical analysis

Differences in mating success between DRO+ and DRO− flies were firstly analyzed using χ^2 tests separately for each sex and temperature. In χ^2 tests, analyses were performed by pooling data from replicated mating cages. We also analyzed the effects of both the fixed stock factor (DRO+ vs. DRO−) and the random replicate factor in a generalized linear model (GLM) with a binomial distribution and logit link function. In addition, differences between thermal treatments were tested with an analysis of the deviances from a Gaussian distribution (best fitted distribution of the data) and logLik link function in a generalized linear model (GLM). Analyses were performed for each sex separately using stock (DRO+ vs. DRO−) and temperature treatment (25 °C, 33 °C and 33 °C-HT) as fixed factors for both sexes. Number of matings was used as dependent variable. LSD Fisher comparisons were performed to test for differences between temperature treatments. To test for mating latency in single-pair assays, GLM analysis with a Gamma distribution and log-link function was performed using DRO+ vs. DRO− and temperature treatment (25 °C vs. 33 °C vs. 33 °C-HT) as fixed factors. Since the stock × temperature interaction was non-significant in all cases, this interaction was removed from the analysis following the principle of statistical parsimony based on the Akaike´s information criterion (AIC, Akaike, 1973; Bates et al., 2015). Analyses were performed using InfoStat software (Di Rienzo et al., 2017). This software implements an interface of the R platform version 3.4.1 (R Core Team, 2017) to estimate generalized linear models through GLM and GLMER procedures from the stats and lme4 libraries (Bates et al., 2013).

3. Results

Mean values for mating success and KRHT are shown in Fig. 1 for DRO+ and DRO− stocks at each temperature and thermal treatment. DRO+ and DRO− stocks differed significantly in their KRHT (ANOVA with [1] stock and [2] sex as a fixed factors: [1] $F_{1,\ 290} = 26.84$ ***; [2] $F_{1,\ 140} = 2.44$; [1] × [2] $F_{1,\ 290} = 3.52$. ***P < 0.001). At 25 °C, there were no significant differences in mating success between heat-resistant (DRO+) and heat-sensitive (DRO−) stocks (Table 1). The same pattern was observed at high temperature, with no significant differences between DRO+ and DRO− stocks in the 33 °C mating experiment without heat hardening, neither in the 33 °C experiment with heat-hardening pre-treatment (Table 1). These results are consistent with both GLM performed considering the random effect of replicate (Table 2) and deviance analysis in GLM of differences between treatments, where the fixed stock factor was not significant (see below).

The total number of matings in the mass-mating cages significantly differed between experimental temperatures (Fig. 2; GLM with [1] stocks DRO+ vs. DRO− and [2] thermal treatment as fixed factors: [1] $\chi^2_1 = 1.62$ for males and $\chi^2_1 = 3.38$ for females; [2] $\chi^2_2 = 197.53$** for males and females; **P < 0.01). In particular, mating success was 1.5 fold lower at 33 °C than at 25 °C in both sexes (LSD Fisher comparisons: $t_{2;\ 46} = 5.36$, $P < 0.05$; Fig. 2). The heat-hardening pre-treatment increased mating success for about 70% at high temperature (Fig. 2). In females, mean mating success was 0.70 folds higher at 33 °C with heat-hardening than at 33 °C without heat-hardening (LSD Fisher comparisons: $t_{2;\ 46} = 2.04$, $P < 0.05$). There was no significant difference in the number of matings between heat-pre-treated and non-pre-treated flies at 25 °C (LSD Fisher comparisons: $t_{2;\ 46} = 2.01$, $P > 0.05$; Fig. 2). In males, mating success with heat hardening did not differ from mating success without heat hardening both at 25 °C (LSD Fisher comparisons: $t_{2;\ 46} = 1.91$, $P > 0.05$) and 33 °C (LSD Fisher comparisons: $t_{2;\ 46} = 1.94$, $P > 0.05$; Fig. 2).

Table 1
Number of DRO+ and DRO− flies engaged in mating in cage experiments performed at 25 °C, 33 °C and 33 °C after a heat hardening pre-treatment (HT). Chi-square values with one degree of freedom were computed to test for differences in the number of flies engaged in mating from the DRO+ and DRO− stocks. P > 0.05 for all comparisons.

Temperature (°C)	Females			Males		
	DRO+	DRO−	χ^2_1	DRO+	DRO−	χ^2_1
25	54	53	0.01	46	61	2.10
33	31	26	0.44	29	28	0.02
33-HT	69	66	0.07	68	67	0.01

Table 2
Generalized linear model performed to test for differences in mating success between DRO+ and DRO− flies in three thermal regimes (see Section 2 for details of the design).

Temperature (°C)	Females			Males		
	df_{effect}	df_{error}	F	df_{effect}	df_{error}	F
25	1	10	0.02	1	10	3.77
33	1	14	0.58	1	14	0.02
33-HT	1	20	0.11	1	20	0.01

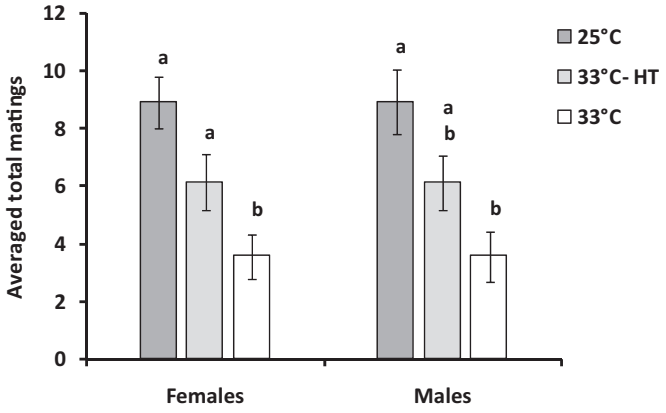

Fig. 2. Averaged number of mating flies (± standard error) over replicates of mating cages and fly stocks (as there were no significant differences between stocks) is shown for males and females at 25 °C, 33 °C and 33 °C after a heat hardening pre-treatment (HT). Mean values with different letter are significantly different according to LSD Fisher comparisons (P < 0.05).

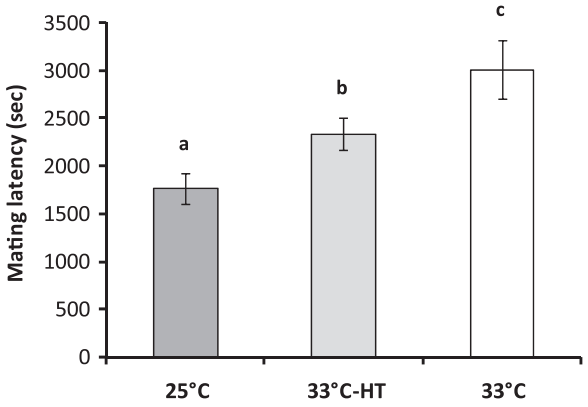

Fig. 3. Mean mating latency (in seconds, ± standard error) in DRO+ and DRO− virgin flies from single-pair assays. Mean values with different letter are significantly different according to LSD Fisher comparisons (P < 0.05).

Mating latency in single pair-assays differed significantly with heat-hardening (Fig. 3; GLM with [1] DRO+ vs. DRO− flies and [2] temperature treatment with vs. without hardening as fixed factors: [1] χ^2_1 = 0.08; [2] χ^2_2 = 13.7***; ***P < 0.001). Flies at 33 °C showed a longer mating latency than flies at 25 °C either with or without heat hardening (LSD Fisher comparisons: $t_{2;\ 287}$ = 2.23* for hardened flies and $t_{2;\ 287}$ = 4.9*** for non-hardened flies; *P < 0.05; ***P < 0.001; Fig. 3). However, heat-hardened flies at 33 °C showed a shorter mating latency than non-hardened flies at this temperature (LSD Fisher comparisons: $t_{2;\ 287}$ = 2.67, P < 0.01; Fig. 3).

4. Discussion

Mating success can be affected by environmental temperature. We tested mating success in contrasting thermotolerance genotypes and temperatures in *D. melanogaster*. No differences between the heat-resistant (DRO+ stock) and heat-sensitive (DRO− stock) genotypes were found for the X-linked QTL of thermo-resistance tested in this study. Consistent differences were found between thermal treatments only. Total number of matings in our mass-mating experiment was significantly lower at high temperature (33 °C) without heat hardening than at benign temperature (25 °C). This is consistent with the results observed for mating latency in single-pair assays, as latency was significantly longer at 33 °C than at 25 °C. Another clear-cut result was that heat hardening strongly improved the short-term mating success at high temperature in both sexes and fly stocks, resulting in no significant differences in total number of matings between heat-hardened flies at 33 °C and non-hardened flies at 25 °C. This beneficial effect of heat hardening is related to a reduction in mating latency by increasing mating speed at high temperature in hardened flies when compared to non-hardened males and females. This is a novel heat hardening effect which was not previously observed in insects.

Strong reduction in mating success due to elevated temperature has been previously observed in *Drosophila* (e.g. Fasolo and Krebs, 2004; Jørgensen et al., 2006; Sambucetti and Norry, 2015). Mating success responds to artificial selection for both culture temperature (Dolgin et al., 2006) and heat resistance (Sambucetti and Norry, 2015). Thermotolerance genotypes have been previously found to affect mating success and other fitness-related traits. For example, mating success and the ability to locate food and reproductive resources at high temperature was affected by the genotype of a thermotolerance QTL in chromosome 2 of *D. melanogaster* (Loeschcke et al., 2011). Differences in mating success were not observed at benign temperatures, indicating that differences were due to the thermotolerance QTL genotype (Loeschcke et al., 2011). In this study, we did not find differences in mating success between the heat resistant and the heat sensitive genotypes for the thermotolerance QTL on chromosome X. In contrast to previously studied QTL on chromosome 2 (Loeschcke et al., 2011), the X-linked QTL in this study appears to be specific of thermotolerance with no effects on mating success at high temperature. Additionally, we tested in an exploratory way any possible correlations between mating success and the expression level of two candidate genes *(hsp60* and *hsc70-3*) within this X-linked QTL (results not shown), both in heat-hardened and non-hardened flies by using expression data for eight RIL-D48 lines from Norry et al. (2009). Although heat knockdown resistance was correlated with a combination of expression levels of both *hsp60 and Hsc70-3* in heat-hardened flies in Norry et al. (2009), mating success as measured in this study was not correlated with expression levels of these two candidate genes (P > 0.05), further supporting that this X-linked thermotolerance QTL does not affect mating success at elevated temperature.

Inducible plastic responses including heat hardening may be crucial for adaptation to changing climatic conditions (Hoffmann and Parsons, 1991; Hoffmann et al., 2003; Hoffmann and Daborn, 2007; Reusch and Wood, 2007; Sgrò et al., 2016). Heat-hardening effects as opposed to heat acclimation are always limited to a short period (e.g., one or few days) in the life of an individual, so a larger effect could be expected for heat acclimation which result from long-term exposure to a sub-lethal heat stress (Hoffmann et al., 2003). Although previous studies have shown a direct response to artificial selection on heat resistance in *Drosophila* (e.g. Krebs and Loeschcke, 1996; Bubliy and Loeschcke, 2005; Sambucetti et al., 2010), it is proposed that many ectotherm species live close to their upper thermal limits (Deutsch et al., 2008; Kellermann et al., 2012). This suggests that the evolutionary response to increase heat resistance might be relatively low, pointing out the role of phenotypic plasticity to mitigate the impact of elevated temperature. Heat hardening can also improve short-term mating success at high temperature, as in the present study. It is well known that the stress response by previous exposure to heat increases the performance for future expositions to elevated temperature (Dahlgaard et al., 1998;

Feder and Hofmann, 1999; Sørensen et al., 2003). This induced protection could be responsible for the better performance in mating success at elevated temperature in heat hardened flies. Hardened flies show a short-term higher mating success than non-hardened flies, which is probably a result of the fact that hardened flies are more active and mate faster than non-hardened flies at elevated temperatures, as in our single-pair assays. Fast-inducible and reversible responses such as heat-hardening, may be more effective and less costly compared to changes in basal resistance and can be evolutionarily favored in unpredictable changing environments (Schilthuizen and Kellermann, 2014; Sørensen et al., 2016; Manenti et al., 2018). Overall, it is apparent that plastic responses can sometimes be stronger than evolutionary and/or genetic responses in determining the mating success at high temperature, but it can strongly depend on each QTL and specific details of the thermal environment. Beneficial effects of heat hardening on short-term mating success such as the observed in this study could also be of interest for the application of sterile insect technique programs under warm conditions, as environmental temperature can affect field performance of mass-reared insects (Loeschcke and Hoffmann, 2007). In the sterile insect technique, released individuals are typically reared and maintained at constant and optimal temperature to optimize rearing productivity regardless of the release environmental conditions. This could impact negatively on the competitiveness of the released insects in the field, particularly if environmental temperature differs from the rearing conditions (Bloem et al., 2004). Thermal pre-treatments prior to release may contribute to a better performance in warm environments, improving competitiveness of released individuals in the field and increasing the effectiveness of the programs to pest control (Chidawanyika and Terblanche, 2011).

5. Conclusions

Reproductive fitness components such as mating success are of interest to test for adaptive responses to thermal stress and for predicting potential evolutionary responses to climate change (e.g., Deutsch et al., 2008; Kellermann et al., 2009; Hoffmann and Sgro, 2011). The present results show for the first time that heat hardening improves short-term mating success at elevated temperature. This plastic response is of ecological relevance in small insects and, as mentioned above, it may also be of interest in sterile insect release programs for the control of pest insects under warm conditions.

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Declarations of interest

Authors declare that they have no conflict of interest.

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